

MARKED UP VERSION OF AMENDMENTS

IN THE SPECIFICATION:

Cancel the paragraph bridging pages 2-3.

Amend the paragraph in lines 12-14 on page 4 to read:

Yet a further aspect of the present invention provides a polypeptide comprising an amino acid sequence selected from [<400>2, <400>4, <400>6, <400>8, <400>10] SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or an amino acid sequence having at least 60% similarity to any one of said sequences.

Amend the paragraph in lines 15-19 on page 4 to read:

Still yet a further aspect of the present invention provides a polypeptide encoded by a nucleotide sequence selected from [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or a[n] nucleotide sequence having at least 60% similarity to any one of said sequences or a nucleotide sequence capable of hybridizing to any one of said sequences under low stringency conditions at 42°C.

Amend the paragraph in lines 25-27 on page 10 to read:

The present is particularly exemplified in relation to *Mycobacterium* antigens B.4, B.6, B.10, MMP and C17 having amino acid sequences and

corresponding nucleotide sequence as set forth in [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 respectively.

Amend the paragraph in lines 1-3 on page 11 to read:

Accordingly, another aspect of the present invention provides a polypeptide comprising an amino acid sequence selected from [<400>2, <400>4, <400>6, <400>8, <400>10] SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10 or an amino acid sequence having at least 60% similarity to any one of said sequences.

Amend the paragraph in lines 4-7 on page 11 to read:

Yet a further aspect of the present invention provides a polypeptide encoded by a nucleotide sequence selected from [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or a[n] nucleotide sequence having at least 60% similarity to any one of said sequences or a nucleotide sequence capable of hybridizing to any one of said sequences under low stringency conditions at 42°C.

Amend the paragraph in lines 8-11 on page 1 to read:

Even yet a further aspect of the present invention provides a nucleotide sequence selected from [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or a[n]

nucleotide sequence having at least 60% similarity to any one of said sequences or a nucleotide sequence capable of hybridizing to any one of said sequences under low stringency conditions at 42°C.

Amend the paragraph in lines 16-21 on page 36 to read:

Nucleotide sequences and corresponding amino acid sequences were determined for antigens B.4, B.6, B.10, MMP and C17 and are shown in [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 respectively.

IN THE CLAIMS:

Cancel claims 10-26, without disclaimer of their subject matter or prejudice to reassertion in this or a continuing application.

Amend claims 1-9 to read:

1. (amended) An isolated polypeptide or a derivative, homologue, analogue, or functional equivalent thereof wherein said polypeptide is obtainable from a species of *Mycobacterium* and which polypeptide is immunointeractive with sera from a human, animal or avian species exposed to said species of *Mycobacterium* or its relative or antigenic parts thereof but which polypeptide is substantially not immunointeractive with sera from a

human, animal, or avian species not [prior] previously exposed to said species of *Mycobacterium* or its relative or its antigenic parts.

2. (amended) The [An] isolated polypeptide according to claim 1 wherein the species of *Mycobacterium* is selected from *Mycobacterium* [is *M.*] *tuberculosis*, *Mycobacterium avium*, *Mycobacterium microti*, *Mycobacterium leprae*, *Mycobacterium lepraeumurium*, *Mycobacterium paratuberculosis*, *Mycobacterium ulcerans*, *Mycobacterium marinum*, *Mycobacterium smegmatis*, *Mycobacterium intracellulare*, *Mycobacterium xenopi*, *Mycobacterium chelonei*, *Mycobacterium fortuitum*, *Mycobacterium farcinogenes*, *Mycobacterium flavum*, *Mycobacterium haemophilum*, *Mycobacterium kansasii*, *Mycobacterium phlei*, *Mycobacterium scrofulaceum*, *Mycobacterium senegalense*, *Mycobacterium simiae*, *Mycobacterium thermoresistible*, and *Mycobacterium xenopi*.

3. (amended) The [An] isolated polypeptide according to claim 2 wherein the species of *Mycobacterium* is *M. tuberculosis*.

4. (amended) An isolated polypeptide or a derivative, homologue, analogue, or functional equivalent thereof wherein said polypeptide is obtainable from *M. tuberculosis* or a related organism and which polypeptide is immunointeractive with sera from a human previously exposed to *M. tuberculosis* or an antigenic extract therefrom but is substantially not

immunointeractive with human sera not previously exposed to *M. tuberculosis* or an antigenic extract thereof.

5. (amended) The [An] isolated polypeptide according to claim 4 wherein the human exposed to *M. tuberculosis* has active pulmonary or extra-pulmonary tuberculosis.

6. (amended) The [An] isolated polypeptide according to claim 4 or 5 wherein the polypeptide has a [molecule] molecular weight of from about 5 kDa to about 100 kDa.

7. (amended) The [An] isolated polypeptide according to claim 6 wherein the molecular weight is selected from about 10 to 20 kDa, 28 to 38 kDa, 38 to 48 kDa, 53 to 63 kDa, and 55 to 65 kDa.

8. (amended) An isolated polypeptide comprising an amino acid sequence selected from [<400>2, <400>4, <400>6, <400>8, <400>10] SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or an amino acid sequence having at least 60% similarity to any one of said sequences.

9. (amended) An isolated polypeptide encoded by a nucleotide sequence selected from [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or a[n] nucleotide

sequence having at least 60% similarity to any one of said sequences or a nucleotide sequence capable of hybridizing to any one of said sequences under low stringency conditions at 42°C.

IMMUNO-REACTIVITIES APPENDIX

Experimental Background

Sera Specimens. A total of 427 human sera specimens were used in this study. Control groups consisted of 178 healthy individuals, 29 individuals with non-TB respiratory disease (ie. lung cancer and asthma), and 109 individuals with inactive TB. All the inactive TB patients exhibited tuberculin skin test positive (PPD +ve), and acid fast stain (AFB) of sputa and bacteria culture negative. The test group consisted of a total of 111 sera specimens from patients with active TB confirmed either by chest X-ray or bacteriologically by culture or sputum AFB smear tests. The extrapulmonary TB sera were confirmed by histological analysis and/or bacteriological culture. These sera specimen were also tested against 2 commercially available TB diagnostic kits, T1 and T1. All sera were aliquoted and stored at -70°C until use.

Immunoblot analysis of recombinant antigens. Western blots were prepared by the conventional SDS-PAGE electrophoresis on Tris-HCl 2D preparative ready-gels (BioRad, Hercules, CA) and subsequently the electrophoresed antigen (10 µg per gel) were Western blotted onto Hybond™-C nitrocellulose membrane (Amersham Life Science, Little Chalfont, United Kingdom) as described previously. After transfer, the membranes were blocked in 5% skimmed milk/TBST, air-dried and stored at 4°C until further use.

The membranes were cut into 3 mm wide strips for testing against the TB sera panels. Screening was carried out in slot trays containing 1 ml of diluted serum specimen (1:100 in 1% skimmed milk/TBST [10 mM Tris, pH 7.5, 300 mM NaCl, 0.005% Tween 20]) per lane, for 1 h with rocking at room temperature (18-25°C). The strips were then washed 4X in TBST followed by incubation with alkaline phosphatase conjugated Goat anti-human Ig (Harlan Sera Lab, Loughborough, United Kingdom) (1:1000 in 1% skimmed milk/TBST) for 1 h with rocking at room temperature. The strips were again washed in TBST (4X), allowed to develop in 1 ml of NBT/BCIP substrate (BioRad) for 4 min and subsequently, the reaction was stopped by washing in ddH₂O (4X). Positive controls consisted of a strip probed with a positive serum specimen, reactive to the recombinant protein antigens, and a second strip probed with the commercially available anti-RGSHis probe (Qiagen). Reactivity of recombinant protein to sera specimens was interpreted based on the density of band obtained on a densitometer, X-Rite® 400 (measuring range 0.00D-2.5D; X-Rite Inc., Grandville, MI).

ELISA assay. Microtiter plates (Medium binding type II EIA strip plate; Corning Costar Corporation, Corning, NY) were coated overnight at room temperature with 100 µl/well of diluted antigen in 0.05 M sodium carbonate buffer, pH 9.6. The optimized coating concentrations per well for the antigens, Antigen Seq. ID 2, 4, 6, 8, 10 and 38-kD were 0.05, 0.065, 0.025, 0.10, 0.025 and 0.029 µg respectively. After two washes with wash buffer [0.05% Tween 20 in phosphate-buffered saline (PBS), pH 7.4], the coated plates were blocked with 200 µl/well of blocking buffer [1% bovine serum albumin (BSA) (Sigma, St Louis. Mo), 2.5% sucrose (Sigma) in sodium

carbonate buffer] for 2 h in room temperature. After incubation, the plates were emptied, dried and subsequently stored at 2-8°C until further use.

For the assay, 100 μ l diluted (1:51) sera specimen with the sample diluent, 1:25 v/v normal Goat Serum (Gibco BRL Life Technologies, Inc., Grand Island, NY), 0.05% Tween 20 (Sigma), 0.02% Thimerosal (Sigma) and 0.3 ml/L phenol (Sigma) in PBS pH 7.4, was added to each well and incubated either for 20 min in room temperature (Seq. ID 8 and 10) or for 1 h in 37°C (Seq.ID 2, B.4, B.6 and 38-kD). After incubation, the wells were washed 3X with 200 μ l wash buffer (0.05% Tween 20, 0.02% Thimerosal and 0.3 ml/L phenol in PBS pH 7.4). In the following step, the wells were filled with 100 μ l of diluted (1:5,000 for Seq. ID 2; 1:6,000 for Seq. ID 4; 1:8,000 for Seq. ID 6 and 8; 1:10,000 for Seq.ID 10; and 1:5,000 for 38-kD antigen) Horseradish peroxidase-conjugated Goat Anti-Human IgG, Fc γ fragment specific (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in a conjugate diluent [1:4 (v/v) Fetal Bovine Serum (Gibco BRL), 1:10 (v/v) Glycerol (Sigma), 0.05% Tween 20, 0.02% Thimerosal and 0.3 ml/L phenol in PBS pH 7.4] and incubated either for 20 min in room temperature (Seq. ID 8 and 10) or for 30 min in 37°C (Seq.ID 2, 4, 6 and 38-kD). The wells were washed 3X after which 100 μ l of one-step TMB (3,3', 5,5'-tetramethylbenzidine) substrate (Dako Corporation, Carpinteria, CA) was added to each wells followed by incubation either for 10 min in room temperature (Seq. ID 8 and 10) or for 15 min at 37°C (Seq. ID 2, 4, 6 and 38-kD). The enzymatic reaction was stopped by the addition of 100 μ l of 1 N sulfuric acid. Absorbance was read within 15 min in an ELISA reader (Spectra, TECAN Austria Ges. m.b.H, Grödig, Austria) at 450 nm with 630 nm as a reference. Blank wells, negative and positive control sera specimens were included in each plate. All the sera specimens were analyzed in duplicate.

Cutoff values. Evaluation of diagnostic specificity for the antigens were based on a positive score represented by antibody titers (interpreted as densitometer units, D, for Western blot and optical density, OD, for ELISA) above the cutoff values calculated based on the means of a control group of healthy individuals (n = 30, BioClinical). On the ELISA assay, the cutoff values were defined as mean OD plus multiples of standard deviations obtained for the control group (Table). The cutoff values were calculated individually for each antigen. With the ELISA format, the number of standard deviations above the mean OD used is shown in footnote in the table. On the Western format, the cutoff were also calculated individually for each antigen, based on the mean of density (D) observed for the 30 normal sera from Bioclinical Inc.

Commercial TB diagnostic kits. As a comparison, all the sera specimens were also screened using two commercially available TB diagnostic kits. An immuno-chromatography-based TB diagnostic kit (T1), ICT Tuberculosis (ICT Diagnostics, Brookvale, NSW, Australia) and an ELISA test kit (T2), PATHOZYME-TB Complex plus (Omega Diagnostics Limited, Alloa, Scotand, United Kingdom) were used.

Statistical Analysis. Assay sensitivities, specificities, positive and negative predictive values were calculated using the statistical software, Win Episcope 1.0 (Borland International Inc., Scotts Valley, CA). Distribution plots were generated using the GraphPad Prism 2.01 software.

The percentage of reactivity for the recombinant TB antigens with different sera specimens as observed on the Western blot and ELISA assays is shown in the Table. Also shown is the percentage of sera specimens detected positive by the commercial TB diagnostic kits (T1 & T2).

Antigen Sequence ID	Theoretical Molecular Weight	Observed Molecular Weight	Healthy Controls (n=178)	Active TB (n=111)	Inactive TB (n=109)	Lung Cancer (n=19)	Asthma (n=10)	PPV ^a	NPV ^b
(a) Western Blot Assay									
Seq. ID 2	55.8	56±3	9.0	29.7	12.8	10.5	10	67.4	67.5
Seq. ID 4	55.0	55±3	8.4	25.2	12.8	5.3	0	65.1	66.3
Seq. ID 6	32.9	33±3	2.2	19.8	4.6	5.3	10	84.6	66.2
Seq. ID 8	16.1	16±3	2.8	27.0	21.1	0	0	85.7	68.1
Seq. ID 10	37.5	38±3	12.9	17.1	7.3	0	0	45.2	62.8
38-kD	NA	NA	16.8	44.1	31.2	10.5	10	62.0	70.5
T1*	NA	NA	7.3	63.1	44.0	10.5	0	84.3	80.1
(b) ELISA assay									
Seq. ID 2	55.8	56±3	6.7	42.3	7.3	10.5	0	79.7	72.2
Seq. ID 4	55.0	55±3	1.7	25.2	4.6	0	0	90.3	67.8
Seq. ID 6	32.9	33±3	6.2	19.0	7.3	0	0	65.6	65.0
Seq. ID 8	16.1	16±3	6.7	35.1	22.0	15.8	0	76.5	69.8
Seq. ID 10	37.5	38±3	12.4	7.2	12.8	5.3	0	26.7	60.2
38-kD	NA	NA	1.7	53.2	18.4	10.5	0	95.2	77.1
T2*	NA	NA	2.2	44.1	30.3	0	0	92.4	73.7

On the Western assay (detecting for human Ig), the cutoff values were: >0.04D for Seq. ID 2 and Seq. ID 4 band; ≥0.04D for Seq. ID 6 and Seq. ID 8 band; ≥0.15D for Seq. ID 10 band; and >0.15D for 38-kD band. On the ELISA assay (detecting for human IgG), the cutoff values were: 0.52 (Mean+4SD) for Seq. ID 2; 2.14 (Mean+3SD) for Seq. ID 4; 0.53 (Mean+4SD) for Seq. ID 6; 0.09 (Mean+2SD) for Seq. ID 8; 0.10 (Mean+3SD) for Seq. ID 10; and 1.06 (Mean+3SD) for 38-kD.

^aPPV, positive predictive value, P >0.05; ^bNPV, negative predictive value, P >0.05

* T1 and T2 commercial kits make use of combinations of 5 and 2 different antigens, respectively.

NA= not applicable.